Evaluation of Puncture Types as Indicators of Boll Weevil (Coleoptera: Curculionidae) Oviposition in Cotton Squares

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ABSTRACT Reproductive boll weevil populations are typically identified by the presence of a frass seal and protuberance at the oviposition site in cotton squares. However, despite the occurrence of other oviposition puncture seal types and their use in previous fecundity studies, the relationship of these respective puncture seal types and oviposition has not been clearly examined. In this study, newly eclosed females (≤24 h old, but mated at 4 d of age) were fed fresh squares daily for 8 d to determine oviposition frequency in relation to individual puncture seal types. Puncture seal types were classified as unsealed; puncture with frass seal; puncture with wax seal; and puncture with wax seal and partially covered with frass. Overall, no significant associations were detected between the types of sealed punctures, and the frequency of oviposition in sealed punctures ranged from 64.6 (wax-seal with frass) to 72.9% (frass-sealed) during 2001 and 53.4 (wax-sealed) to 55.2% (wax-seal with frass) during 2002. Examination of individual trials revealed considerable variability in oviposition associated with all sealed puncture types. Oviposition also occurred in unsealed punctures by mated females in all trials. Because of the high degree of oviposition observed in punctures not normally associated as oviposition sites (i.e., wax-seal with frass, wax-seal), this study clearly shows the need to consider other puncture types as potential indicators of reproductive weevil populations. These results will be critical in boll weevil management programs where accurate detection of reproductive weevil populations is crucial for continued pest suppression.

KEY WORDS boll weevil, oviposition, puncture types, cotton

The boll weevil, *Anthonomus grandis grandis* Boheman (Coleoptera: Curculionidae), continues to be a significant pest of cotton despite ongoing and successful coordinated programs for weevil eradication. Although boll weevil captures in traps determines a need for treatment, field assessments of cotton flower buds (squares) can be used to estimate the presence of a reproductive population. These assessments typically rely on the accurate identification of boll weevil oviposition sites on squares. It is commonly accepted that oviposition sites in field situations are characterized by a protuberance (or proliferation of tissue) at punctures typically sealed with frass.

In a study examining the relationship of sealed punctures to estimate boll weevil egg laying and fecundity, Everett and Ray (1962) documented various types of boll weevil punctures and concluded that sealed punctures showed a "good correlation" as a measure of oviposition. However, Everett and Ray (1962) were unclear whether the documented puncture types were observed in their laboratory study, but methods suggest the primary focus included punctures sealed with frass. Although providing more detailed descriptions of oviposition punctures, Greenberg et al. (2003) also relied on sealed punctures as indicators of oviposition sites to measure weevil fecundity without dissecting the squares. Conversely, Showler (2004) dissected squares to determine oviposition but did not clearly define criteria for determining oviposition sites. Thus, the occurrence of boll weevil punctures with different seal types has been previously observed (Hunter and Hinds 1905, Cushman 1911, Everett and Ray 1962, Greenberg et al. 2003, 2004). However, none of these earlier studies confirmed the reliability of these different seal types on punctures as indicators of oviposition. Because the cotton industry generally assumes that punctures sealed by boll weevils indicate oviposition (Greenberg et al. 2004), the objective of this study was to evaluate the association between different boll weevil puncture types and oviposition. This data will improve the accuracy in determining the presence of reproductive boll weevils in cotton fields.

Materials and Methods

Source of Boll Weevils. Known-age boll weevils were reared from field-collected infested squares harvested directly from cotton plants in commercial fields near College Station, TX. Collected squares were held in 20.3

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by 20.3 by 20.3-cm Plexiglas cages under controlled environmental conditions (constant 29.4 ± 1°C; 13:11 L:D). After ≈5 d, squares were checked for completion of larval development and presence of pupae. Pupae were removed from the squares, placed in a petri plate containing moistened vermiculite, and returned to conditions described above. Petri plates containing ≈35 pupae were checked twice daily for adult eclosion. Newly eclosed adults were sexed using the tergal notch method of Sappington and Spurgeon (2000).

Experimental Design. For each trial during 2001 and 2002, newly eclosed females (20–30 ♀♀/trial, 2001; 30 ♀♀/trial, 2002) were held and fed individually in petri plates (100 by 15 mm). To provide females with a fresh food source daily, cotton (DP 436 RR; Delta and Pine Land Co., Scott, MS) was grown in a greenhouse. Each female received one unadulterated square (6–9 mm with bracteoles intact) per day for 8 d. Squares of this size are preferred by ovipositing female weevils (Greenberg et al. 2004). A 10-mm length of dental roll soaked in distilled water provided a water source in each plate. Water was replenished as needed. Squares were replaced daily, and removed squares were examined under a dissecting scope (SZ60; Olympus, Kalamazoo, MN) for characterizing puncture types. Based on preliminary observations, puncture types were classified as unsealed; puncture sealed with frass (frass-seal); puncture sealed with wax film (wax-seal); and puncture sealed with wax film but partially covered with frass (wax with frass-seal). After puncture characterization, a razor blade was used to dissect each square to determine the presence of eggs in relation to individual puncture types. Six and five independent trials were conducted during 2001 and 2002, respectively.

To ensure females would oviposit while held in petri plates, known-age males were also reared for mating. Males were held in two 473.18-ml cartons (30 males/carton), and weevils were fed debracted squares at the rate of one square per two weevils during each trial. Each carton also contained a 22.2-ml plastic water vial with a cotton dental wick extending through the lid. Maturation of the sexual organs for adult boll weevils has been reported to occur at ≈3 d (Cushman 1911); thus, 4-d-old males were introduced to 4-d-old females after removal of squares from petri plates on the fourth feeding day. Two males were provided per female. Weevils were monitored ≤4-h period, and pairs exhibiting mating behavior were examined under the microscope to confirm insects were in copula. After the ≤4-h mating period, males were removed, and females were again provided fresh squares daily for an additional 4 d. Cohorts of eggs corresponding to each female were held in individual petri plates containing moistened filter paper to confirm egg fertilization. Egg hatch was not calculated.

Data Analyses. Virgin females cannot produce fertile eggs; therefore, the 4-d feeding period for virgin females was classified as unmated (1–4 d of age), and the subsequent 4-d feeding period was classified as postmated (5–8 d of age). Although puncture types presumably reflect oviposition by mated females, puncture records were also noted during the premating period.

Table 1. Overall percentage (n) of individual boll weevil puncture types with indicated seals that contained eggs for six and five trial runs during 2001 and 2002, respectively

Female status (age)	Puncture seal type	Year 2001	Year 2002
Unmated (1–4 d)	Frass	9.5 (21)[a]	1.3 (78)[a]
	Wax with frass	0.0 (3)[a]	—
	Wax	0.0 (2)[a]	5.6 (18)[a]
	Unsealed	0.0 (2,106)	0.1 (1,825)
Mated (5–8 d)	Frass	72.9 (708)[b]	53.5 (787)[b]
	Wax with frass	64.6 (82)[b]	55.2 (308)[b]
	Wax	74.5 (572)[b]	53.4 (723)[b]
	Unsealed	1.6 (2,141)	5.4 (1,720)

[a] Within female category and year, nonsignificant association between sealed punctures and frequency of oviposition (2001, Fisher's exact, P = 1.00; 2002, Fisher's exact, P = 0.34); unsealed punctures excluded from analyses but data are presented for comparison.

[b] Within female category and year, nonsignificant association between sealed punctures and frequency of oviposition (2001, χ^2 = 3.55, df = 2, P = 0.17; 2002, χ^2 = 0.32, df = 2, P = 0.85); unsealed punctures excluded from analyses but data are presented for comparison.

The TABLES statement of the PROC FREQ procedure (SAS Institute 2001) was used to evaluate the association between puncture type and oviposition. In the contingency table analyses, puncture types were the rows and presence/absence of eggs were the columns. The ALL and FISHER options of the TABLES statement were used to generate the χ^2 and Fisher exact test statistics, respectively, and these were used where appropriate to determine significant relationships. For each year, data for all trial runs were pooled for analyses, but variability in individual trials was also examined using the BY statement of the PROC FREQ procedure. Additionally, in an effort to relate this oviposition data to previous literature (Everett and Ray 1962), all sealed puncture types were pooled, and this sealed puncture category was compared with unsealed punctures. Analysis of variance (ANOVA) using PROC GLIMMIXED (SAS Institute 2001) was explored, and overall results were similar to those generated by PROC FREQ. However, in consultation with a statistician and considering the binary nature of the data, it was determined that the PROC FREQ analyses would be more informative than an ANOVA procedure.

Results

For unmated females, unsealed punctures were predominant during both years (Table 1) and indicate significant feeding activity by newly eclosed adults. However, oviposition was observed in one of these unsealed punctures during 2002 (Table 1). When the unsealed punctures were excluded from the analyses, no significant association was observed between the frequency of egg presence and sealed puncture type during 2001 (Fisher exact, P = 1.00) or 2002 (Fisher exact, P = 0.34) for unmated females (Table 1).

For mated females, unsealed punctures continued to be predominant during both years (Table 1), and the number of sealed punctures and frequency of

Table 2. Distribution and percentage (*n*) of punctures by mated female boll weevils (5–8 d old) that contained eggs in respective trials during 2001 and 2002

Year	Puncture seal type	Trial run					
		1	2	3	4	5	6
2001	Frass	71.1 (90)	67.6 (179)	78.1 (164)[a]	76.8 (155)	66.7 (48)	72.2 (72)
	Wax with frass	54.6 (11)	71.4 (28)	40.0 (10)[a]	72.0 (25)	33.3 (3)	80.0 (5)
	Wax	76.9 (65)	72.3 (94)	75.9 (133)[a]	78.6 (154)	61.0 (41)	71.8 (85)
	Unsealed	0.7 (297)	1.9 (318)	2.7 (369)	1.1 (367)	0.3 (365)	2.6 (425)
2002	Frass	70.4 (169)[b]	57.2 (166)	43.4 (152)	52.0 (131)	43.2 (169)[b]	—
	Wax with frass	66.2 (77)[b]	51.0 (51)	60.7 (28)	58.3 (60)	44.6 (92)[b]	—
	Wax	45.3 (137)[b]	52.5 (141)	55.2 (69)	54.6 (141)	58.1 (179)[b]	—
	Unsealed	5.4 (353)	3.1 (350)	2.6 (390)	4.9 (328)	12.0 (299)	—

[a] Within year, there was significant association between sealed puncture type and frequency of oviposition for trial 3 only (χ^2 = 7.46, df = 2, $P < 0.02$). There were no significant associations in remaining trial runs.

[b] Within year, there was significant association between sealed puncture type and frequency of oviposition for trial 1 (χ^2 = 21.35, df = 2, $P < 0.0001$) and trial 5 (χ^2 = 8.88, df = 2, P = 0.01). There was no significant associations in remaining trial runs.

oviposition increased. When the unsealed punctures were included in the analyses for mated females, a significant association was observed between puncture seal type and egg presence (2001, χ^2 = 2,052.74, df = 3, $P < 0.0001$; 2002, χ^2 = 981.94, df = 3, $P < 0.0001$). However, in both years, this association was caused by the occurrence of fewer than expected unsealed punctures with eggs. A small percentage of unsealed punctures containing eggs was observed during both years (Table 1).

When unsealed punctures were excluded from analyses for mated females, no significant association was observed between the frequency of egg presence and puncture type during either year when trials were pooled (2001, χ^2 = 3.55, df = 2, P = 0.17; 2002, χ^2 = 0.32, df = 2, P = 0.85; Table 1). During 2001, the overall percentage of sealed punctures containing eggs ranged from 64.6 (wax with frass-seal) to 74.5 (wax-seal). Lower overall percentages of sealed punctures with eggs were observed during 2002 and ranged from 53.4 (wax-seal) to 55.2 (wax with frass-seal). Except for the wax with frass-seal category, approximately similar numbers of punctures and punctures with eggs were observed between years. The cause for the discrepancy in observed frequencies of sealed punctures with eggs between years is unknown. Because the pattern and number of punctures for puncture category during 2002 was relatively consistent with 2001 data (Table 1) and identical methods were used during the study, the author can only speculate that the reduced frequency of oviposition during 2002 was a biological phenomenon.

While the initial analyses of pooled trials indicates lack of significant association between sealed puncture types and oviposition (Table 1), examination of data for the independent trial runs indicated considerable variability in the frequency of oviposition associated with sealed punctures ranging from 33.3 to 80.0% (Table 2). Additionally, significant associations between sealed puncture types and oviposition frequency were observed for one and two trials during 2001 and 2002, respectively, and these were being masked by the initial analyses. However, it is likely that the observed numbers of punctures for these trials influenced the statistical significance and these observations may be biologically insignificant for these three trials. During both years, a general trend was observed of more frass-sealed and wax-sealed punctures (Table 2). More importantly, oviposition was also observed in unsealed punctures for all trials.

To compare this study with previous work by Everett and Ray (1962), sealed puncture types were pooled for comparison with unsealed punctures, and a significant association was observed between the frequency of seal presence and oviposition for unmated and mated females (Table 3). Oviposition seems to be related to the presence of sealed punctures, but the observed variability suggests seal presence is not an absolute measure.

Table 3. Percentage (*n*) of sealed and unsealed punctures observed in the laboratory as estimators of boll weevil oviposition

Female status (age)	Puncture seal type[a]	Year	
		2001	2002
Unmated (1–4 d)	Sealed	7.7 (26)[b]	2.1 (96)[b]
	Unsealed	0.0 (2,106)[b]	0.1 (1,825)[b]
Mated (5–8 d)	Sealed	73.1 (1,362)[c]	53.7 (1,818)[c]
	Unsealed	1.6 (2,141)[c]	5.4 (1,720)[c]

[a] Sealed puncture category includes punctures sealed with frass; punctures sealed with wax film; and punctures sealed with wax film and partially covered with frass.

[b] Within female category and year, there was a significant association between puncture type and frequency of oviposition (2001, Fisher's exact, $P < 0.0001$; 2002, Fisher's exact, P = 0.01).

[c] Within female category and year, there was a significant association between puncture type and frequency of oviposition (2001, χ^2 = 2,049.37, df = 1, $P < 0.0001$; 2002, χ^2 = 981.57, df = 1, $P < 0.0001$).

Discussion

Extremely low oviposition was observed in sealed punctures produced by virgin females, and this agrees with previous observations (Mayer and Brazzel 1963). The observations of ovipositing virgin females contribute to the importance of accurately determining oviposition sites because rarely is the mating status of feral females known, and sealed punctures produced by mated females are indistinguishable from those produced by virgin females. Observations in this study included estimates of oviposition in unsealed punctures that have been previously classified as feeding sites and further support the need for square dissections to accurately estimate oviposition.

Although the frequency of oviposition in unsealed punctures was lower than in sealed punctures, these data suggest unsealed punctures cannot be discounted as oviposition sites. The high number of unsealed punctures is not surprising because developing females obviously need resources for survival and egg production. Indeed, unsealed punctures have been traditionally classified as feeding sites (Greenberg et al. 2003). Records for individual females indicate that oviposition in unsealed punctures was not an artifact in this study. Oviposition occurred in squares where females only produced unsealed punctures. Additionally, where a mixture of sealed and unsealed punctures were produced on a square, all sealed punctures contained eggs but some unsealed punctures contained eggs as well. The relatively consistent frequency of oviposition in sealed and unsealed punctures suggest that observations of unsealed punctures could be potential precursors to detection of reproductive weevil populations. That is, despite the absence of a traditional indicator of an oviposition site (i.e., protuberance at the puncture site), a developing weevil population may exist in the area. Everett and Ray (1962) speculated that development of the protuberance is a result of squares remaining on the plant. Because squares were removed and dissected daily in this laboratory study, the protuberance commonly associated with an oviposition site in the field was not observed. Temporal observations of punctured squares remaining on cotton plants indicate the protuberance may require up to 72 h to develop (unpublished data).

Everett and Ray (1962) were proponents of using sealed punctures over dissection of squares as a labor-efficient method for estimating oviposition despite previous observations that unsealed puncture sites contained eggs (Cushman 1911). Additionally, after counting sealed punctures and dissecting the same squares in their labor-efficiency trial, calculations of values provided by Everett and Ray (1962) indicated that only 60% of sealed punctures ($n = 219$) contained eggs, although they concluded that sealed punctures were reliable estimators of fecundity. Based on the conclusions of Everett and Ray (1962), a researcher would erroneously presume oviposition in all sealed punctures, but data presented in this study prove otherwise.

In this study, the overall frequency of oviposition in sealed punctures by mated females ranged from 53.4 to 74.5%. Calculations of observed oviposition frequencies identified puncture types for combined years indicate frass-sealed and wax-sealed punctures exhibited similar oviposition rates ($\approx$62%). Wax with frass-sealed punctures exhibited slightly lower oviposition rates (57%) for combined years. One could argue that these observations are similar to the estimate provided by Everett and Ray (1962), but examination of individual trial runs in this study also indicates the occurrence of a low percentage of oviposition (33.3%) in sealed punctures. Granted this low percentage is based on a low number of observed punctures, but it is plausible that low numbers of sealed punctures may be encountered in the field, especially in regions that have reached advanced stages, or completion, of boll weevil eradication. In these instances, the results presented here are critical to management of boll weevils.

This study has clearly identified boll weevil puncture types and oviposition rates associated with the respective puncture types. Results indicate that sealed punctures do not necessarily reflect oviposition and show the need to exercise caution in solely using frass-sealed punctures as indicators of boll weevil oviposition. Because accurate determination of oviposition is a critical component of boll weevil management programs, these data suggest researchers should consider the presence of other puncture types as indicators of oviposition in field situations and future boll weevil fecundity studies. Failure to consider other boll weevil puncture types in field situations may result in resurgence of boll weevil populations.

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